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What Reaches the Antenna? How to Calibrate Odor Flux and Ligand–Receptor Affinities

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Abstract

Physiological studies on olfaction frequently ignore the airborne quantities of stimuli reaching the sensory organ. We used a gas chromatography–calibrated photoionization detector to estimate quantities released from standard Pasteur pipette stimulus cartridges during repeated puffing of 27 compounds and verified how lack of quantification could obscure olfactory sensory neuron (OSN) affinities. Chemical structure of the stimulus, solvent, dose, storage condition, puff interval, and puff number all influenced airborne quantities. A model including boiling point and lipophilicity, but excluding vapor pressure, predicted airborne quantities from stimuli in paraffin oil on filter paper. We recorded OSN responses of *Drosophila melanogaster*, *Ips typographus*, and *Culex quinquefasciatus*, to known quantities of airborne stimuli. These demonstrate that inferred OSN tuning width, ligand affinity, and classification can be confounded and require stimulus quantification. Additionally, proper dose–response analysis shows that *Drosophila* AB3A OSNs are not promiscuous, but highly specific for ethyl hexanoate, with other earlier proposed ligands 10- to 10 000-fold less potent. Finally, we reanalyzed published *Drosophila* OSN data (DoOR) and demonstrate substantial shifts in affinities after compensation for quantity and puff number. We conclude that consistent experimental protocols are necessary for correct OSN classification and present some simple rules that make calibration, even retroactively, readily possible.

Key words: DoOR, electrophysiological recordings, olfaction, photoionization detector, receptor, vapor pressure

Introduction

Many insect behaviors are mediated by volatile stimuli that are detected by olfactory sensory neurons (OSNs) found primarily in the antennae and maxillary palps. To know which compounds insects respond to, electrophysiological recordings and behavioral assays are performed. In such studies, synthetic chemicals are typically diluted in a solvent and applied on some sort of dispenser from which the odor molecules evaporate. During electrophysiological recordings, odor-laden filter papers are often the dispenser and are positioned inside Pasteur pipettes (e.g., Larsson et al. 2001; Hallem and Carlson 2006; Andersson et al. 2009; Bengtsson et al. 2009; Hill et al. 2009; Carey et al. 2010). During stimulation, an air puff is passed through the pipette, and the molecules in the headspace are transported into a continuous airflow that passes over the antenna. Commonly, many compounds are tested for physiological responses in the insect. Comparison among the responses is confounded, however, as the compounds often have dramatically different evaporation rates (Bengtsson et al. 1990; Hartlieb and Rembold 1996). The lack of measurement of vapor concentration or stimulus flux means that the number of molecules presented to the insect is unknown as it depends on the identity of the compound. Thus, true response specificities and sensitivities of receptors, neurons, or antennae are difficult to derive (Mayer 1993).

Evaporation of a compound from such a stimulus cartridge dispenser depends not only on its vapor pressure (VP), but also on its affinity to the solvent and filter paper. Even compounds of the same molecular weight, such as monoterpene hydrocarbons, may have different evaporation rates in the same solvent (Brockerhoff and Grant 1999). In general, it is assumed that the vapor concentration of a compound is linearly proportional to the liquid concentration, but deviations from the rule exist, especially at the higher concentrations >1–10% v/v (Cometto-Muñiz et al. 2003) that are not uncommon to OSN classification (Ghaninia et al.

2007; Hill et al. 2009). The relationship between liquid and vapor concentrations is also solvent dependent; compounds evaporating from paraffin oil exhibited a linear relationship, whereas compounds in hexane did not (Brockerhoff and Grant 1999). Thus, the use of various solvents and liquid concentrations makes comparisons between studies tenuous (Cometto-Muñiz et al. 2003; Tsukatani et al. 2003). These incongruities have sparked the development of a consensus database of odor responses (DoOR; http://neuro.uni-konstanz.de/DoOR/default.html) for *Drosophila melanogaster* (Galizia et al. 2010). Although representing a useful database, DoOR is based upon responses to compounds of unknown amounts reaching the OSNs.

While it is not common practice to quantify airborne stimuli in physiological studies, researchers have been aware of the problem (e.g., Hebets and Chapman 2000; Olsson et al. 2010), and techniques for quantification do exist; for instance by headspace sampling followed by gas chromatographic (GC) analysis (Jang et al. 1989; Bengtsson et al. 1990; Hoskovec et al. 1993; Hartlieb and Rembold 1996; Cossé et al. 1998; Brockerhoff and Grant 1999; Meijerink and van Loon 1999; Ochieng et al. 2002; Syed and Leal 2008). However, the GC approach becomes exceedingly time consuming if vapor amounts of many compounds across numerous successive stimulations must be measured. Thus, a fast and direct method may often be needed for efficient quantification. Photoionization detectors (PIDs) have become increasingly deployed in olfactory research (Riffell et al. 2008). They have been used for concentration control in an olfactometer (Johnson et al. 2003), to measure plume structure in the field (Andersson et al. 2011) and in the lab (Justus et al. 2002; Dekker et al. 2005), as well as antennal response properties of moths in turbulent pheromone plumes (Justus et al. 2005) and of *Drosophila* in a wind tunnel electroantennogram (EAG) setup (Schuckel et al. 2008). A PID has also been used for development of odor delivery systems (French and Meisner 2007; Olsson et al. 2010) and to determine how the temporal structure of odor stimuli is affected by the physical parameters of the odor delivery system (Vetter et al. 2006). In the latter study, airflow, tube diameter, distance from tube exit, distance from odor source, and lateral distance from tube axis all affected the temporal integrity of the stimulus.

We used a PID to measure the release of 27 compounds (C2–C10; most of plant origin) from standard Pasteur pipette stimulus cartridges. We verified how airborne quantities released from stimulus cartridges are affected by the compound, puff number, liquid concentration, solvent, and storage of the cartridge. We subsequently analyzed which physical explanatory variables (particularly VP) could be used to accurately compensate for headspace quantities. Finally, using 3 insect model species (the bark beetle, *Ips typographus,* the Southern house mosquito, *Culex quinquefasciatus*, and the fruit fly, *D. melanogaster*), we assessed if and how differences in volatilization rates could influence physiological studies characterizing OSN types and affinities.

Materials and methods

The photoionization detector

We used a ppbRAE+ PID (model PGM7240; RAE systems Inc.) equipped with a 10.6 eV lamp for measurements of odor concentrations in air. This detector uses a UV light source to ionize airborne molecules. The ions produce a charge that is measured by the instrument sensor (Application Note AP-226, rev 2 wh. 01-04, RAE systems Inc.). The PID measures concentrations down to the low ppb range but at a relatively low sampling rate of 1 Hz. Whereas other types of PID instruments allow higher time resolution (Rouyar et al. 2011), the instrument we used offers a higher sensitivity, which was crucial for characterization of the less volatile compounds at physiologically relevant doses. The pump creates a flow of 600 mL/min through the detector. The detector was calibrated with isobutylene gas at 10 ppm concentration. We used the peak value of the PID response to an odor puff as the measure of airborne concentration, as it was more consistent than using area integration.

Calculation of response factors

The ppb readings of the PID are contingent on the sensitivity of the instrument to the various compounds. Although a calibration list is available for relative calibration (Technical Note TN-106, rev 15 wh. 1-06, RAE systems Inc.), few of the compounds used in our study were on that list, and our aim was to calibrate for conversion into absolute amounts. We therefore verified the sensitivity of the PID to our panel of odorants. A Gerstel MPS-2 autosampler (Gerstel GmbH, Muelheim and der Ruhr; microoven temperature 90 °C) collected 2 mL headspace from a 20-mL headspace vial containing 1% of a compound in paraffin oil. Half of this sample was injected into a 500 mL/min airstream through the PID and the other half injected into a GC with a flame ionization detector (FID) (Agilent 6890a, DB-wax column, inlet 220 °C, program: 40 °C for the initial 2 min, then rising by 10 °C/min up to 220 °C).

We subsequently analyzed the sensitivity of the FID to the compound by injecting 100 ng of the compound in hexane into the GC-FID. One unit of the PID reading thus corresponded to

$$\mathrm{mol}_{\mathrm{PID}} = \frac{A_{\mathrm{FID}}}{\mathrm{ppb}_{\mathrm{PID}}} \frac{10^{-7}}{C \cdot M.}$$

In which $\mathrm{mol}_{\mathrm{PID}}$ = one unit of PID reading (mol), A_{FID} = FID area value of the split headspace injection (area units), $\mathrm{ppb}_{\mathrm{PID}}$ = photoionization peak value measurement in the split headspace injection (ppb, unit less), C = calibration value of the FID (i.e., FID area value with injection of 100 ng, area g^{-1}), and M = molar mass of the compound (g mol^{-1}). The split headspace injections into the PID and

GC-FID, as well as the 100-ng injections were repeated to verify the consistency of the measurements ($N = 3$).

Stimuli and odor delivery system

Odor stimuli in aliquots of 10 μL were loaded on Whatman 3 filter paper (cat. no. 1003-150, cut to strips of 1.5 × 0.5 cm, Whatman, Ltd.), positioned inside standard Pasteur pipettes. Paraffin oil (product # 1.07162.1000, MERCK) was used as the standard solvent. To compare amongst different solvents, some compounds were diluted also in pentane (Fluka, >99%). For odors in paraffin oil, pipettes were capped with a plastic pipette tip (1 mL) immediately after stimulus application. Pipettes loaded with compounds diluted in pentane were left uncapped for 5 min to allow for evaporation of the pentane to which the PID responds. It was not practical to use hexane as solvent due to the high sensitivity of the detector and the relatively long (compared with pentane) evaporation time.

Charcoal filtered airflow was provided by stimulus controller CS-55 (Syntech). Two air outlets were used, the pulse and complementary pulse, the latter delivering continuous airflow between stimulations at 700 mL/min to the PID. The pulse and complementary systems were connected by Teflon tubes (inner diameter 5 mm) and a T-connection that directed a single airflow toward the PID. During odor stimulation, a stimulus pipette was connected to the pulse system and a 2-s air pulse at 700 mL/min was passed through the stimulus pipette and onto the PID. Simultaneously, the flow from the complementary system was shut off, ensuring a constant airflow to the PID. The inlet of the PID was positioned approximately 0.5 cm into the tube, without creating a tight seal. At least 1 min passed between odor stimulations enabling remaining molecules to escape from the system. Control stimulations with empty pipettes between odor stimulations demonstrated that responses were not affected by previous stimulations.

Depletion

The depletion during successive stimulations over time was measured for 27 compounds (Table 1). The experimental protocol was designed to mimic a full day of electrophysiological recordings using pipettes loaded once before the onset of the experimental session. The first stimulation was performed 30 min after loading for compounds in paraffin. Each pipette was puffed 50 times (or until the compound was below response threshold, whichever came first) with 10 min between puffs (i.e., 8 h and 10 min between the 1st and 50th stimulation). Pipettes were kept at room temperature (RT) throughout the experiment. All compounds diluted in paraffin were tested at 100 μg on the filter paper. Compounds with a high vapor concentration were tested also at 10 μg and some at 1 μg. Depletion for compounds diluted in pentane was studied only at the 100 μg dose. For compounds in pentane, the first puff from each pipette was discarded from analysis because occasionally, the pentane blank gave a response. After the first puff, we never obtained responses to pentane solvent blanks.

Depletion data were analyzed using linear regression (SPSS 16.0) on the dependent (y: airborne odor amount) and independent (x: puff number over time) variables. Regression models with $\log(y + 1)$ against x, as well as $\log(y + 1)$ against $\log(x)$, were also tested. For compounds reaching values ≤10 ppb, regression analysis only included stimulations prior to the first ≤10 ppb stimulation. The slope coefficient from the regression analysis was used as the measure for depletion rate. Linear regression was subsequently performed to test how accurately the airborne quantity at puff 1 predicted the empirically derived compound depletion rates (i.e., depletion rate slope coefficients in Table 1 used as dependent variable). A similar regression was done to test if VP alone (or log VP) accurately predicted depletion rates.

Finally, a multiple regression analysis, again with depletion rate as dependent variable, including VP, molar mass, number of carbon atoms, boiling point, and lipophilicity as independent variables, was performed because VP alone did not accurately predict the depletion rate. All VPs are at 25 °C and derived from www.chemspider.com.

As a measure of lipophilicity (approximately the inverse of polarity), we calculated (in ChemSketch V.12, ACD/Labs) the log *Poct/wat* for all compounds. *P*, the partition coefficient, is a solute concentration ratio between 2 solvents, in this case, 1-octanol and water. Negative values indicate a higher concentration in water than in octanol.

Accumulation and storage

Five compounds, covering the range of measured depletion rates, were used to measure headspace accumulation after stimulus loading and its depletion during storage. Pipettes were loaded, kept at RT, and puffed once after a log series of 2, 4, 8, 16, 32, 60, 120, 240, 480, 960, 1440 (1 day), and 2880 min, respectively. For the longer storage times (240–2880 min), pipettes stored at –20 °C were also tested. These pipettes were placed at RT 20 min before stimulation. All compounds were dissolved in paraffin and tested at the 100 μg dose on the filter paper.

Physiology

To investigate whether differences in volatilization and in previous use of stimulus pipettes could affect OSN classification, we retested the physiological responses of OSNs on 3 different insect models, using single sensillum recordings (SSR). Different from previous work, we calculated the absolute airborne amounts we used for stimulation. First, we tested the OSN of the European spruce bark beetle, *I. typographus*, that responds best to 3-octanol (Andersson et al. 2009). Apart from 3-octanol, we included 1-octen-3-ol, *Z*3-hexen-1-ol, and 1-hexanol, which are secondary odorants for this OSN. The response to fresh pipettes was compared with the response to pipettes that had been puffed 10 times

Table 1 Chemicals used and the regression values of depletion rates for compounds in paraffin oil at the 100 μg dose on a filter paper

Compound	Source	Purity (%)	ppb → pmol[b]	Regression depletion rate[c]				C atoms	M (g/mol)	VP (Pa)[a]	Boiling point (°C)	Lipophilicity (log *Poct/wat*)
				Type (*y*-*x*)	R^2	Slope	Intercept					
Ethanol	Solveco	99	42.3	log-log	0.84	−1.77	4.8	2	46	11041	78	−0.19
2-Methyl-3-buten-2-ol	Acros	99	3.46	log-log	0.86	−2.47	5.0	5	86	3053	99	0.87
1-Butanol	Scharlau C	99	15.7	log-log	0.91	−2.64	5.4	4	74	1136	118	0.88
Hexane	Fluka	99	11.4	log-lin	0.93	−0.321	5.1	6	86	20131	69	3.94
Ethylbutyrate	Aldrich	99	5.53	log-lin	0.97	−0.125	4.9	6	116	1859	121	1.77
2-Hexanone	Fluka	96	2.52	log-lin	0.94	−0.111	4.7	6	100	1773	128	1.44
3-Hexanone	Aldrich	98	3.44	log-lin	0.96	−0.115	5.0	6	100	1613	123	1.44
Hexanal	Aldrich	98	4.83	log-lin	0.97	−0.108	4.8	6	100	1453	122	1.97
*E*3-hexen-1-ol	Aldrich	98	1.76	log-lin	0.96	−0.078	4.7	6	100	139	157	1.61
*Z*3-hexen-1-ol	Acros	98	1.97	log-lin	0.98	−0.079	4.8	6	100	139	157	1.61
2-butoxyethanol	Fluka	99	1.83	log-lin	0.97	−0.064	4.4	6	118	74	161	0.80
Cyclohexanol	Aldrich	99	2.68	log-lin	0.98	−0.074	4.9	6	100	117	171	1.34
1-Hexanol	Fluka	99	9.85	log-lin	0.96	−0.071	5.0	6	102	126	152	1.94
Methyl hexanoate	Aldrich	99	4.53	log-lin	0.97	−0.042	4.4	7	130	527	195	2.30
4-Methylcyclohexanol	Fluka	98	2.99	log-lin	0.96	−0.036	4.6	7	114	63	172	1.83
1-Octen-3-ol	Acros	98	0.99	log-lin	0.97	−0.022	3.9	8	128	71	175	2.64
Ethyl hexanoate	Aldrich	99	2.68	log-lin	0.89	−0.021	4.0	8	144	221	168	2.83
(−)-α-Pinene	Fluka	99	0.82	log-lin	0.92	−0.020	4.0	10	136	465	155	4.37
Hexylacetate	Aldrich	98	5.58	log-lin	0.89	−0.016	4.0	8	144	185	169	2.83
3-Octanol	Acros	99	2.56	log-lin	0.97	−0.015	4.1	8	130	68	175	2.82
Myrcene	Fluka	95	0.68	log-lin	0.88	−0.014	3.6	10	136	305	165	4.58
1.8-Cineole	Aldrich	99	0.85	log-lin	0.74	−0.0094	3.6	10	154	220	177	2.82
(+)-Limonene	Fluka	99	1.08	log-lin	0.70	−0.0081	3.6	10	136	205	176	4.45
Methyl octanoate	Acros	99	2.67	log-lin	0.83	−0.0064	3.3	9	158	70	151	3.37
Methyl salicylate	Sigma	99	0.69	log-lin	0.67	−0.0056	2.7	8	152	9	222	2.23
(−)-Linalool	Fluka	98	0.57	lin-lin	0.79	−12.5	3.2[d]	10	154	12	199	3.28
1-Octanol	ICN	98	9.19	lin-lin	0.84	−73.5	3.9[d]	8	130	15	195	3.00

The last 5 columns represent candidate explanatory variables for the depletion rate. *Note 1*: Compounds are listed according to Figure 1A, that is, at which puff concentration is 50% of concentration at first puff for compounds in paraffin oil. *Note 2*: The shading highlights the ranking of each variable, as well as the ranking of compounds in terms of depletion rate (see also text).

[a]VPs at 25 °C derived from www.chemspider.com.

[b]Factor to convert the response of the detector (part per billion) into airborne stimulus amount (picomoles).

[c]Slope coefficients for compounds with different transformation (none or log) of dependent (*y*) and independent (*x*) variables are not directly comparable. For examples of regressions on different scales, see Figure 2.

[d]Log-transformed intercepts for comparison with the other compounds.

Please note, this table appears in color in the online version of *Chemical Senses*. The color version of this table provides a better visual representation of Table 1's values than the grayscale version reproduced above.

before with a 2-min stimulus interval (''old'' pipettes). Compounds were presented in random order, but the 2 pipettes with the same compound were always tested pairwise (old pipette first). For each pipette, the PID response was recorded before and after stimulating the antenna, and the before–after average was used to correlate the PID and OSN responses. The applied dose on the filter paper was 10 μg. Two minutes elapsed between PID recordings and physiological recordings using the same pipette. At least 1 min passed between successive OSN stimulations with different pipettes.

To compare the sensitivity of the PID with the sensitivity of insect OSNs and to determine OSN dose–response characteristics based on airborne amounts, we studied OSN responses of *I. typographus*, the southern house mosquito, *C. quinquefasciatus*, and the fruit fly, *D. melanogaster*. For the beetle, we tested the cell that selectively responds to 2-methyl-3-buten-2-ol (MB) (Andersson et al. 2009). This cell was also chosen to check whether the low number of MB cells reported in the previous study could be the result of the high volatility of the compound, that is, if cells might have been nonresponsive due to lack of MB in the headspace. For the mosquito, we tested the short sharp trichoid 3A neuron (SST3A; Hill et al. 2009), using 1-octen-3-ol, 2-butoxyethanol, and 4-methylcyclohexanol (compounds previously identified as the top 3 ligands), as stimuli. In testing both of these OSNs, we used a single stimulus pipette for each compound diluted in paraffin and alternately stimulated the PID and the OSN (starting with the PID) 25 times each with 1 min between successive stimulations. The applied dose on the filter paper was 10 μg for the beetle and 100 μg for the mosquito. The before–after average PID measurement was used to correlate the OSN response to the PID

response. After the last stimulation, an odor puff from a fresh pipette was delivered to the insect to ensure that the neuron had not fatigued during the test period. The continuous airflow was 1.3 L/min and the odor pulse was 700 mL/min during 2 s. Stimulation conditions for the PID measurements are described above under "Stimuli and odor delivery system."

SSR verification in *D. melanogaster* was performed on the AB3A neuron, which is one of the best-studied OSNs in insects (Stensmyr et al. 2003; Pelz et al. 2006), and therefore an important reference point. Different from the recordings on the beetle and the mosquito, stimulations were performed "concentrations up" through $\log_{10}$ dilution of stimulus headspace samples using a Gerstel MPS-2 autosampler (see also under "Calculation of response factors"). A major reason for the different protocols was the high sensitivity of the neuron to hexanoates, which results in extremely tonic physiological responses at higher hexanoate concentrations, followed by a period of adaptation (decreased sensitivity to lower concentrations). In addition, AB3A neurons are many log magnitudes more sensitive to hexanoates than the PID, which would make PID measurements of little value unless the stimulus headspace could be diluted in a reliable manner. Log_{10} dilution steps with the MPS-2 autosampler were perfectly linear across 5 log concentrations (measured with MB as tracer odor). Using a 2.5-mL headspace syringe, the MPS-2 sampled a fraction of the headspace from a 20-mL vial containing a 10^{-2} dilution of an odor in paraffin oil. After serial dilution of the headspace with ambient air within the syringe of the MPS-2, odors were injected (duration 2 s) into a heated injection port (150 °C), connected to a 1 L/min airstream leading over the fly antenna. Stimulus concentrations were determined using the PID and calibrated using the correction factors for each compound (see above under "Calculation of response factors").

Of all 3 species, females were used in recordings. Electrophysiological equipment (Syntech), insect preparation, and odor delivery system are described previously (Stensmyr et al. 2003; Andersson et al. 2009; Hill et al. 2009). OSN responses were calculated offline in AutospikeTM 3.0 (Syntech) by counting the number of spikes (action potentials) during the initial 500 ms of the response and subtracting the spontaneous activity during the 500-ms prestimulation period. Responses were then converted (i.e., doubled) into spike frequency (Hz).

Reanalysis of OSN affinities in *D. melanogaster*

To assess how odor quantity may affect interpretation of OSN affinities, we reanalyzed a subset of *D. melanogaster* OSNs (AT3: Or19a, AC3: Or35a, AB3B: Or85b, unknown: Or85c), the response of which have been compiled across studies in DoOR (Galizia et al. 2010), a publicly available database (www.uni-konstanz.de). To assess the potential influence of volatilization and depletion rate, we compensated for the airborne stimulus amount. First, the DoOR response intensity (RI) to a given odor X was converted to a putative OSN response in hertz. An RI of 1.0 was converted to an OSN response of 260 Hz, as this response frequency appeared, in our study, to be the maximum of the *D. melanogaster* AB3A OSN to key ligands. Then, we derived the corresponding stimulus quantity, using our regression equation derived from our AB3A neuron's dose–response to its key ligand ethyl hexanoate. This we did for all ligands for a given Or for which we had experimental data. Subsequently, we compensated for differences in headspace quantity between these ligands using our PID-derived experimental values. This was done by calculating a ratio in headspace quantity between this odor and the odor in our panel that according to the DoOR database gives the highest RI. This ratio was then used to compensate for the presumed difference in airborne amounts between the 2 stimuli. Next, the new amount was fed into the AB3A dose–response regression equation to derive a new RI. This was done for those *Drosophila* Ors for which we had a reasonable number of own experimental data. The adjusted RIs are thus based on an equal number of molecules in the headspace.

In addition, because the DoOR database is based on an unknown number of stimulations per cartridge, we also assessed whether "age" (previous puffing) of the cartridge could, in theory, also affect the hierarchy of ligands. For that we used our PID measurements of the 1st, 10th, and 20th successive stimulation with the same stimulus pipette. Note that with the above calculations, we verified only the potential effect of depletion rates and age of the cartridge on estimated ligand affinities in *Drosophila*, for which it is necessary to assume a similar dose–response slope for all compounds and OSNs. However, different regression slopes are commonly found, especially between good and poor ligands, which further affect inferences about true ligand affinities (i.e., the scores on ligand affinity are likely conservative estimates).

Results

Depletion of headspace

The depletion rate experiment (100 μg in paraffin oil) revealed a wide spectrum of release rates across the compounds tested (Figure 1A). The decline in airborne amounts during successive stimulations with the same pipette of the 3 most volatile compounds, ethanol, 1-butanol, and MB, was best described by a log(amount)–log(puff#) linear function (Table 1 and Figure 2A). The airborne amount of these compounds dropped dramatically between the 2 initial stimulations. The depletion of most other compounds was best described by a log(amount)–linear(puff#) function, although there was a large variation in depletion rates among these compounds (Figures 1A and 2B,C). In contrast, the

airborne amount of linalool and 1-octanol decreased linearly (Figure 2D). There was a clear linear relationship between the amount released from a stimulus pipette at puff 1 and the subsequent depletion rate over successive stimulations (Figure 3A).

Several physical parameters (i.e., VP, molar mass, lipophilicity, number of carbon atoms, and boiling point) appeared to affect compound release from stimulus cartridges. However, the shading in Table 1 illustrates the low accuracy of any of these parameters alone in predicting the sequence of the empirically derived depletion rate. If a certain parameter accurately predicted the relative depletion rate of a compound in relation to the rest of the panel, the shading would follow the gradient of the measured depletion rate column. However, if the parameter overestimates or underestimates the depletion rate of that compound, the cell is darker or lighter, respectively, than the corresponding cell in the depletion rate column. None of the parameters alone aligned satisfactorily with the experimental data, which implies that none accurately predicted the depletion of stimuli over reiterative stimulations. In particular, VP, which is generally assumed to be the best predictor of compound volatilization, showed only a moderately linear relation with depletion rate (R^2 = 0.55; Figure 3B).

In contrast, deploying a multiple regression using all the independent (non-orthogonal) physical variables (VP, molar mass, number of C atoms, lipophilicity, and boiling point), we obtained a model in which only boiling point and lipophilicity contributed significantly ($F_{2,23}$ = 73.3, $P < 0.0001$, R^2 = 0.86) to the dependent variable depletion rate (from Table 1):

$$Y = -0.010(\pm 0.002)BP - 0.178(\pm 0.043)L + 0.519(\pm 0.205).$$

In which $Y = \log_{10}$(depletion rate), BP = boiling point, and L = lipophilicity (SI units as in Table 1). Interestingly, the standardized regression coefficient β (a measure of effect size or importance for the relation) was –0.62 for BP and –0.40 for L, indicating a larger importance for BP.

In addition, factors such as functional group, saturation, and position of double bond may affect the rate of depletion but are probably included in L. For the ten C6 compounds, one may directly compare variation due to functional group and saturation (Table 1). Pipettes loaded with ketones and aldehydes were depleted at a higher rate than the corresponding alcohols. Unsaturated alcohols were depleted at a higher rate than the saturated alcohols. The monoterpene hydrocarbons had the highest lipophilicity and were depleted

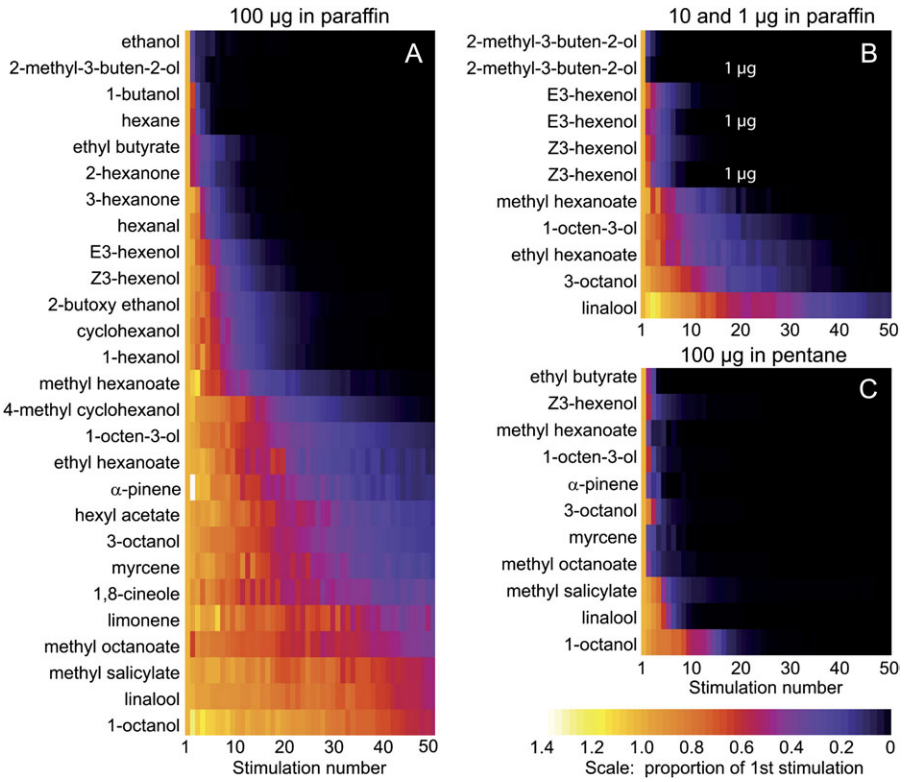

Figure 1 Depletion of compounds in paraffin oil at (**A**) a 100 µg dose on the filter paper (N = 4) and (**B**) 2 lower doses, 10 and 1 µg (3 compounds indicated in graph, N = 3–4), represented in heat plots. Compounds are listed according to the puff at which airborne amount was 50% of the amount at the first puff. For all compounds, setting the average initial response to 1.0 normalized the responses and other responses are expressed as proportions of this value. (**C**) Depletion of compounds diluted in pentane at a dose of 100 µg on the filter paper (N = 4). Please note, this figure appears in color in the online version of *Chemical Senses*.

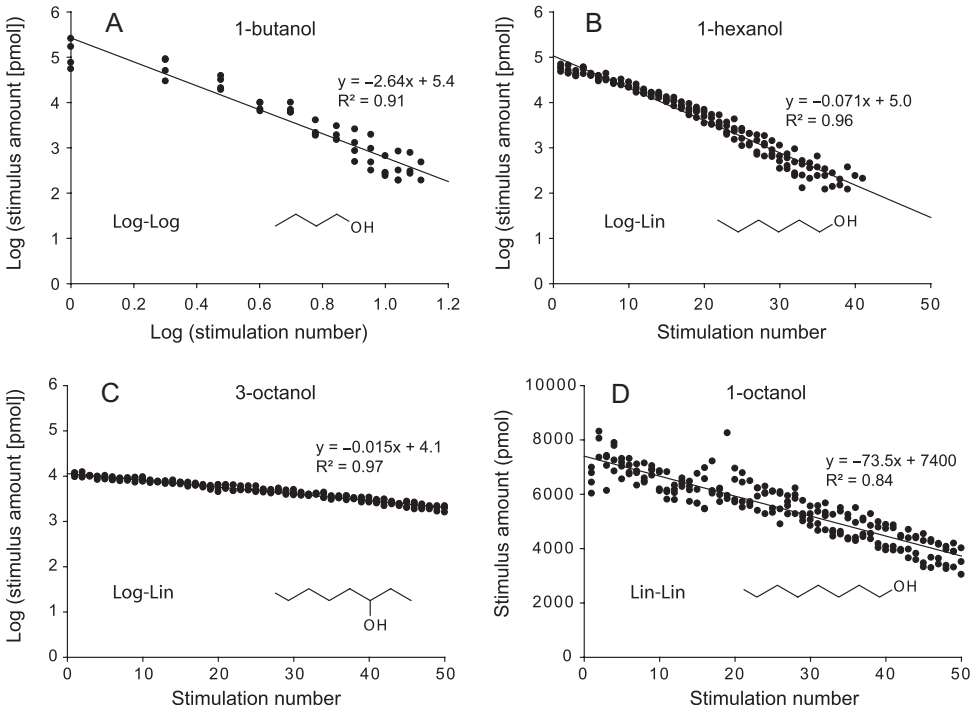

Figure 2 Regression analyses for 4 representative compounds demonstrating different rates and functional forms of depletion of airborne amounts. Compounds were dissolved in paraffin oil at a 100 μg dose on the filter paper (N = 4). Note the different scales for the dependent and independent variables in **A**–**D**. (A) The decrease of 1-butanol was best described by a log(y + 1)–log(x) function. 1-Hexanol (B) and 3-octanol (C) had different depletion rates, but both were best described by a log(y + 1)–linear(x) function. (D) The low depletion rate of 1-octanol was best described by a simple linear function.

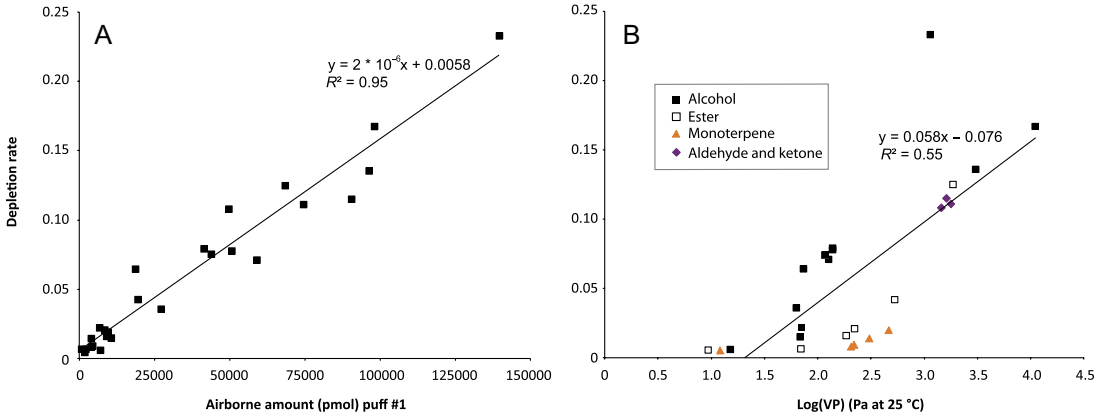

Figure 3 (**A**) Relation between the airborne amount given off by stimulus pipettes at the first puff and the depletion rate (from Table 1) for compounds in paraffin oil at a 100 μg dose on the filter paper. (**B**) A positive relation between log-transformed VP and depletion rate was found, however, several compounds are far from the line, indicating that other factors influence volatilization and depletion of compounds dissolved in paraffin oil (see also results from multiple regression analysis). In general, monoterpene hydrocarbons and esters fall below the line, whereas alcohols are more spread out. The outlier hexane was excluded from analyses in both A and B. Please note, this figure appears in color in the online version of *Chemical Senses*.

at a lower rate than predicted by their VPs (Figure 3B) due to their affinity for the paraffin oil solvent.

Vapors of the more volatile compounds were reliably detected (>100 ppb) by the PID down to the 1 μg dose on the filter paper. Regression analysis on log-transformed doses and PID responses was used to determine linearity of the PID over the tested range of doses (1, 10, and 100 μg on the filter paper). Regressions for all compounds (slope coefficients: 0.87, 1.06, and 1.02 for MB, *E*3-, and *Z*3-hexenol, respectively) demonstrated an almost completely linear dose–response of the detector and a very good fit to the regression model ($P < 0.001$; $R^2 > 0.95$).

The relative rate of depletion of compounds from pipettes also depended on applied dose on the filter paper; lower

doses were in general depleted (proportionally) faster than higher doses, as indicated by different regression slope coefficients (Figures 1B and 4A–C). For *E*3- and *Z*3-hexenol, the difference between doses was significant, as factorial analysis of variance on log-transformed amounts (in picomoles) demonstrated a significant interaction between applied dose and stimulation number (*E*3-hexenol: $F_{25,190} = 8.4$, $P < 0.001$; *Z*3-hexenol: $F_{22,192} = 13.4$, $P < 0.001$). The depletion rate for the 100 µg dose on the filter paper was lower than for the 2 lower doses (1–10 µg; Figure 4B,C). For MB, the interaction between dose and stimulation number was weak ($F_{7,58} = 0.53$, $P > 0.05$; Figure 4A).

The solvent type strongly affected depletion rates. As expected, all compounds depleted faster when diluted in pentane (Figure 1C). Apart from 1-octanol, which had a -log(amount)–linear(puff#) depletion rate in pentane, airborne amount of all compounds were rapidly reduced, following a log(amount)–log(puff#) function (Table 2). Solvent type also affected the order of depletion rates among compounds, which is illustrated by the shading in Table 2. If the depletion from pentane-dissolved compounds followed a similar sequence as those dissolved in paraffin, the shading of the paraffin column would be aligned with the pentane column, which is clearly not the case. For instance, pipettes loaded with monoterpene hydrocarbons or methyl octanoate, which had low or intermediate depletion rates in paraffin, were depleted very quickly when dissolved in pentane (Figure 1A,C). The intercepts from regression analysis of especially ethyl butyrate, α-pinene, and methyl hexanoate were 10- to 100-fold smaller when dissolved in pentane, as compared with paraffin oil (Tables 1 and 2). This suggests a significant loss of compound before first puff and/or during the

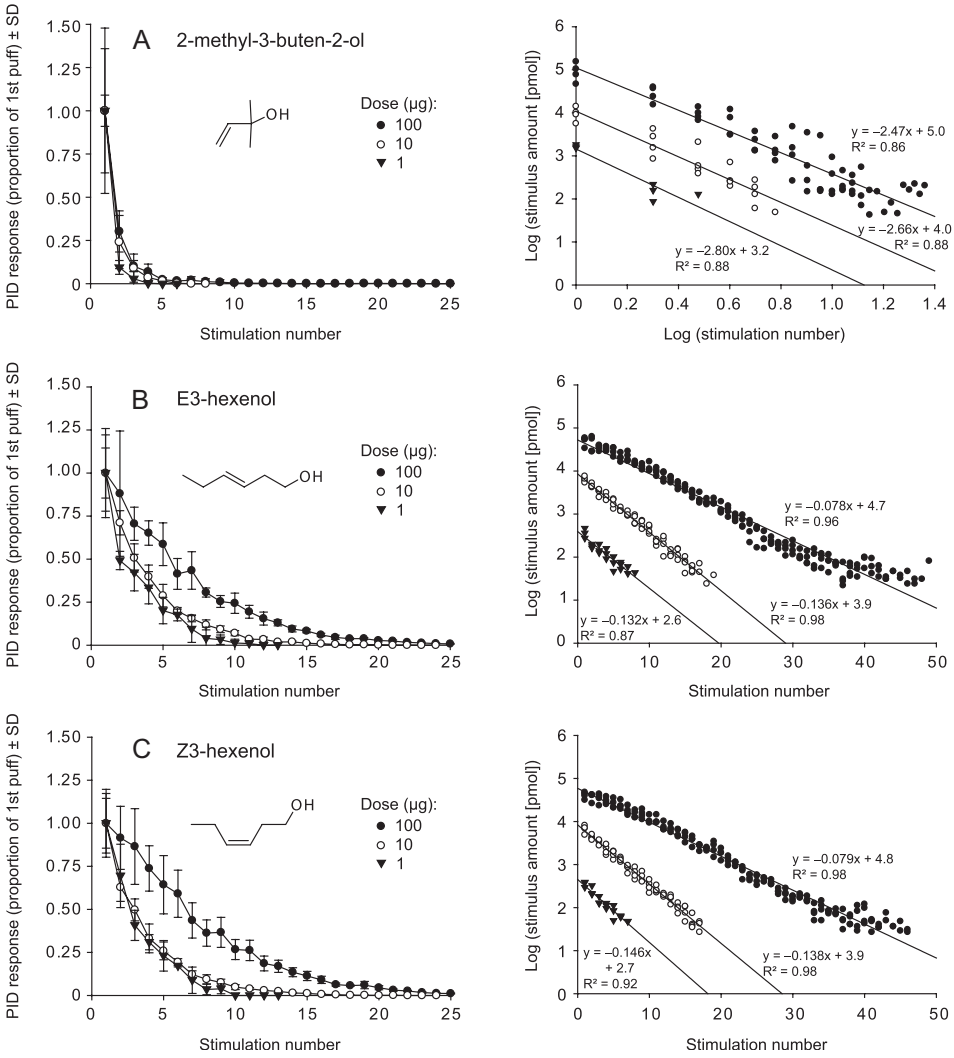

Figure 4 Response of the PID to successive stimulations of 3 doses (1, 10, and 100 µg on filter paper, $N = 3$–4) of compounds in paraffin oil, plotted both as normalized responses (proportion of first stimulation, left column) and as linear regressions on log-transformed airborne amounts (right column). (**A**) MB (stimulation numbers also log transformed for this compound), (**B**) *E*3-hexenol, and (**C**) *Z*3-hexenol. Note: regression slopes for MB are not directly comparable to slopes of the C_6 alcohols due to log transformation of the independent variable (stimulation number) in A.

first puff (which we did not record, as occasionally pentane blanks elicited PID responses in the first puff). The large first puff loss lowers the regression slope coefficient and causes low R^2 values for the highly volatile compounds (Table 2). In contrast, the less volatile compounds have higher concentrations at the initial recorded puffs, which subsequently may result in a steeper slope for the depletion rate. Thus, Figure 1C may depict depletion rates in pentane better than the slope coefficients in Table 2. The high concentrations at the initial puffs of the less volatile compounds, such as the C8 alcohols, methyl octanoate, and especially methyl salicylate and linalool, are reflected in the intercepts from regression analysis, which were 10- to >100-fold larger when dissolved in pentane (Tables 1 and 2). These compounds had depletion rates in pentane comparable to the most volatile compounds dissolved in paraffin. The 3 compounds with the lowest depletion rates in pentane (i.e., methyl salicylate, linalool, and 1-octanol) showed only a slight decrease in airborne amount during the initial 5–10 puffs (depending on compound), followed by a rapid decline (Figure 1C).

Accumulation and storage

Accumulation and loss of odor headspace during storage depended on compound identity and storage temperature. At RT, in spite of the fact that the stimulus pipettes were capped at the pipette's large aperture end, concentrations of MB declined significantly within 240 min after loading and were depleted within 2 days (Figure 5A). For *Z*3-hexenol and methyl hexanoate, headspace concentrations started to decline much later and retained roughly 1% and 10%, respectively, of the maximum concentration over a 2-day period (Figure 5B,C). In contrast, headspace concentrations of 3-octanol and methyl salicylate increased significantly over 2 h after loading and declined by only approximately 40% over a 2-day period (Figure 5D,E).

At –20 °C there was also a significant reduction in concentration of MB over time, albeit delayed compared with RT storage (Figure 5A). In contrast, headspace concentrations of *Z*3-hexenol and methyl hexanoate did not decrease at –20 °C, whereas those of 3-octanol and methyl salicylate increased over time (Figure 5B–E).

Single sensillum recordings

The importance of headspace quantification on OSN classification was assessed using 3 model species: the bark beetle *I. typographus*, the mosquito, *C. quinquefasciatus*, and the fruit fly, *D. melanogaster*. In addition, a cross-species analysis of sensitivity thresholds was performed.

Bark beetle

To test whether reiterative use of stimulus pipettes could affect classification of OSNs, we analyzed the cell responding best to 3-octanol in *I. typographus*. Using newly prepared (fresh) stimuli, the 2 strongest OSN responses were elicited by 3-octanol and *Z*3-hexenol (Figure 6). A slightly weaker response was recorded to 1-octen-3-ol and an even weaker response to 1-hexanol. Ten puffs (i.e., old pipettes) reduced airborne amount mostly for the C6 compounds. This resulted in a completely different response spectrum of the OSN, with 3-octanol as the single best ligand, followed by 1-octen-3-ol that still elicited a relatively strong response. The responses to *Z*3-hexenol and 1-hexanol were now clearly weaker than to the C_8 alcohols.

Using MB, the sensitivity of the PID was compared with the sensitivity of the *I. typographus* OSN specific for MB. The response of the PID dropped much quicker during successive

Table 2 Depletion rates of compounds dissolved in pentane at a 100 µg dose on a filter paper and comparison with depletion rates of compounds in paraffin (from Table 1)

Compound	Regression depletion rate (pentane)[b]				Paraffin	C atoms	M (g/mol)	VP (Pa)	Boiling point (°C)	Lipophilicity (log *Poct/wat*)
	Type (*y*-*x*)	R^2	Slope	Intercept	Slope (log-lin)					
Ethylbutyrate[a]	log-log	0.53	−1.18	3.0	−0.125	6	116	1859	121	1.77
Methyl hexanoate	log-log	0.63	−1.71	3.4	−0.042	7	130	527	151	2.30
Myrcene	log-log	0.65	−1.83	2.9	−0.014	10	136	305	165	4.58
Methyl octanoate	log-log	0.96	−2.07	4.8	−0.0064	9	158	70	195	3.37
Methyl salicylate	log-log	0.96	−2.09	4.8	−0.0056	8	152	9	222	2.23
*Z*3-hexenol	log-log	0.93	−2.14	4.7	−0.079	6	100	139	157	1.61
3-Octanol	log-log	0.95	−2.62	5.5	−0.015	8	130	68	175	2.82
(−)-α-Pinene[a]	log-log	0.42	−2.66	3.0	−0.020	10	136	465	155	4.37
1-Octen-3-ol	log-log	0.96	−2.74	5.1	−0.022	8	128	71	175	2.64
(−)-Linalool	log-log	0.88	−3.03	5.7	−12.5[c]	10	154	12	199	3.28
1-Octanol	log-lin	0.84	−0.076	4.9	−73.5[c]	8	130	15	195	3.00

Shading of columns as in Table 1 (see also text).
[a]Regression analysis on initial 3–4 stimulations, after which concentration was below detection threshold.
[b]Slope coefficients for compounds with different transformations (none or log) of the independent variable (puff number) are not directly comparable.
[c]Slope coefficients from linear regression (not log–lin).
Please note, this table appears in color in the online version of *Chemical Senses*. The color version of this table provides a better visual representation of Table 2's values than the grayscale version reproduced above.

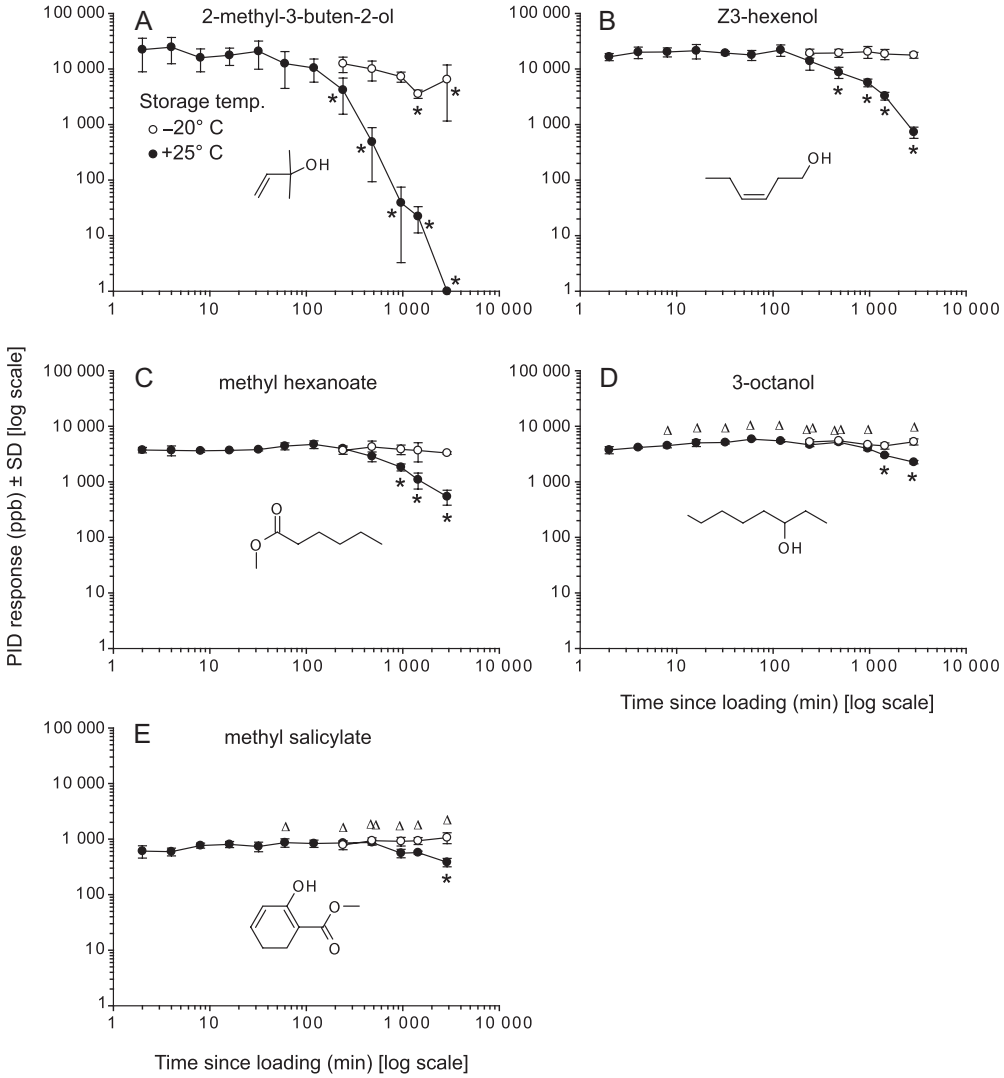

Figure 5 PID response to stimuli (100 μg in paraffin oil on the filter paper) puffed at various time points after loading and stored at different temperatures ($N = 4$). (**A**) MB, (**B**) *Z*3-hexenol, (**C**) methyl hexanoate, (**D**) 3-octanol, and (**E**) methyl salicylate. Concentrations that are significantly lower ($P < 0.05$; one-way analysis of variance on $\log_{10}$-transformed PID responses, followed by the Dunnett's test) than the concentration at 2 min are indicated by *. Concentrations significantly higher than the concentration at 2 min are indicated by Δ. For clarity, different significance levels are not indicated, and pairwise tests were done only to compare with the concentration at 2 min.

stimulations with a single cartridge than the response of the OSN (Figure 7A). At the point where the PID response reached zero (puff# 9 into the PID), the OSN still responded at 112 Hz (ca. 65% of the response to the first puff). The OSN still responded at puff# 25 (i.e., 50 puffs in total). All MB cells here were located in a restricted area, that is, on the lateral borderline between the "B" and "C" bands of sensilla on the antenna, as reported earlier (Andersson et al. 2009).

Mosquito

Similar to the MB cell of the beetle, the response to 1-octen-3-ol by the PID dropped much quicker than did the response of the SST3A neuron of *C. quinquefasciatus* (Figure 7B,E). At the final puff, the PID response reached only 5% (225 ± 33 pmol) of the first puff, yet the OSN responded to 1-octen-3-ol at 42 Hz (ca. 40% of the response to the first puff). However, for the less potent ligands, 2-butoxyethanol (Figure 7C,E) and, especially 4-methylcyclohexanol (Figure 7D,E), the reduction in OSN response followed the reduction in PID response more closely. For 2-butoxyethanol, only 1% of the PID response and 17% of the OSN response remained at the final puff (Figure 7C). In the case of 4-methylcyclohexanol, the trend is inverted; the response to 2125 ± 422 pmol at puff #16 (16% of first puff) was reduced to a nonsignificant response of 1.5 Hz (Figure 7D). The response of the OSN to the most potent ligand tested, 1-octen-3-ol, is a log(concentration)–linear(response) relationship ($R^2 = 0.97$), whereas for the 2 less potent ligands, the relationships are linear(concentration)–linear(response) ($R^2 = 0.92$ for both 2-butoxyethanol and 4-methylcyclohexanol).

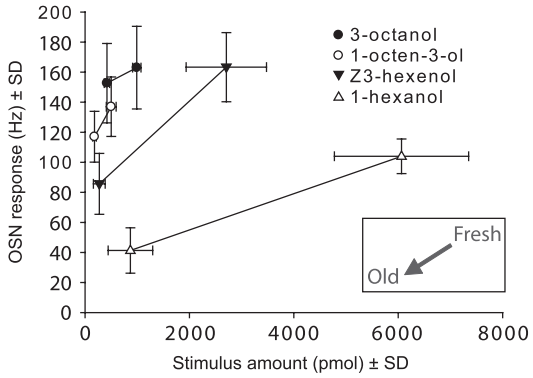

Figure 6 Response of the 3-octanol OSN of *Ips typographus* to airborne amounts of 4 compounds. Responses elicited by an old (i.e., puffed 10 times before, left) and a fresh (not puffed before, right) pipette containing the same compound are connected with a line ($N = 6$).

Fruit fly

Recordings from *D. melanogaster* demonstrate that AB3A neurons respond to a wide variety of compounds at high concentrations (Figure 8). However, at low concentrations, the neuron exhibits strong affinities only for hexanoates. Figure 8 shows that several tens of femtomoles induce clear sensory responses. A further 100× dilution (i.e., fractions of femtomolar quantities) of ethyl hexanoate still induced an increased spiking rate (data not shown). After compensating for headspace quantity, ethyl butyrate becomes a rather poor ligand with a more than 10 000× lower potential to induce OSN responses compared with the key ligand ethyl hexanoate. Interestingly, all odors induced log(concentration)–linear(response) relationships, although the poorer the ligand, the lower the slope coefficient. Key ligand regressions (ethyl and methyl hexanoate) show that almost all variation in OSN responses is explained by ligand concentration ($R^2 = 0.95$). R^2 values were lower for poorer ligands ($R^2 = 0.18$–0.93; Figure 8).

Sensitivity thresholds

Headspace quantification also enables the assessment of OSN sensitivity thresholds within and between species. A comparison across our 3 models illustrates that threshold sensitivities of the cells can vary by at least a million, with fly AB3A neurons responding to fractions of femtomolar quantities (Figure 8) of ethyl hexanoate (and possibly comparable sensitivities of the bark beetle MB cells; Figure 7A,E), whereas mosquito SST3A neurons responded to nanomolar headspace amounts of 1-octen-3-ol (Figure 7B,E).

Comparison with DoOR Data

We subsequently assessed the potential significance of depletion and volatilization in literature studies using the data available in the DoOR database (Galizia et al. 2010, http://neuro.uni-konstanz.de/DoOR/default.html). A limited number of Ors were assessed, namely those that respond to several stimuli of our odor panel.

To assess the importance of the depletion rate on assignment of ligands, the responses were fitted to the equation derived for the response of AB3A neurons to ethyl hexanoate (see "Materials and methods"; Figure 8). Figure 9 demonstrates the effect of compensation for volatilization and depletion rates on DoOR data using our experimentally derived values for these compounds. If compensation for headspace quantity had no effect, the shading in the 3 columns (1st, 10th, and 20th stimulation) would follow the shading derived from the DoOR data (1st column). The data demonstrate that the hierarchical assignment of stimuli based on the RI can shift substantially after compensation for evaporation and depletion rates. For example, the best ligand for Or19a in DoOR, 1-octen-3-ol may actually be second best after compensation for airborne quantity, with linalool being better. Other seemingly unimportant ligands (ethyl butyrate and 1-butanol) may be important if the database was based on relatively older pipettes (10–20 stimulations, when headspace concentrations of very volatile compounds have been reduced to a low level due to repeated puffing), but of lesser importance if only fresh pipettes were used. The best ligands for Or35a may not be *Z*3-hexenol and 1-hexanol if compensated for headspace quantities, but may shift to 1-octanol if DoOR responses were based on measurements with only fresh pipettes or 1-butanol with older pipettes. Other compounds also showed significant shifts in hierarchy. Using our calculations, Or85b may have better ligands than *E*3-hexenol, namely methyl hexanoate, 1-octen-3-ol, or hexanal, depending on the previous puffing of the pipette used. The data clearly demonstrate that both stimulus quantity and stimulation number need to be considered when classifying and evaluating OSN affinities.

Discussion

Much research in olfaction is devoted to characterization of early olfactory processes, such as characterization of OSN repertoires, their individual threshold sensitivities, tuning curves, as well as the biochemical factors (e.g., odor-binding proteins) and physical chemistry factors (solubility of odors in the mucus or lymph and the sensillar lipid layer in insects) that interplay with OSN characteristics. However, a study of these olfactory intricacies requires estimates of the odor flux over the OSN. Such estimates are very tedious and often need to be performed (semi)offline. Hence as a rule, these estimates are not made, although researchers are aware of the problem this may cause for accurate interpretation of the results. Our laboratory is no exception, and we investigated the extent to which such "informed ignorance" can affect results and conclusions, and whether we can provide a fast online means to overcome the current difficulty in stimulus quantification in olfactory research.

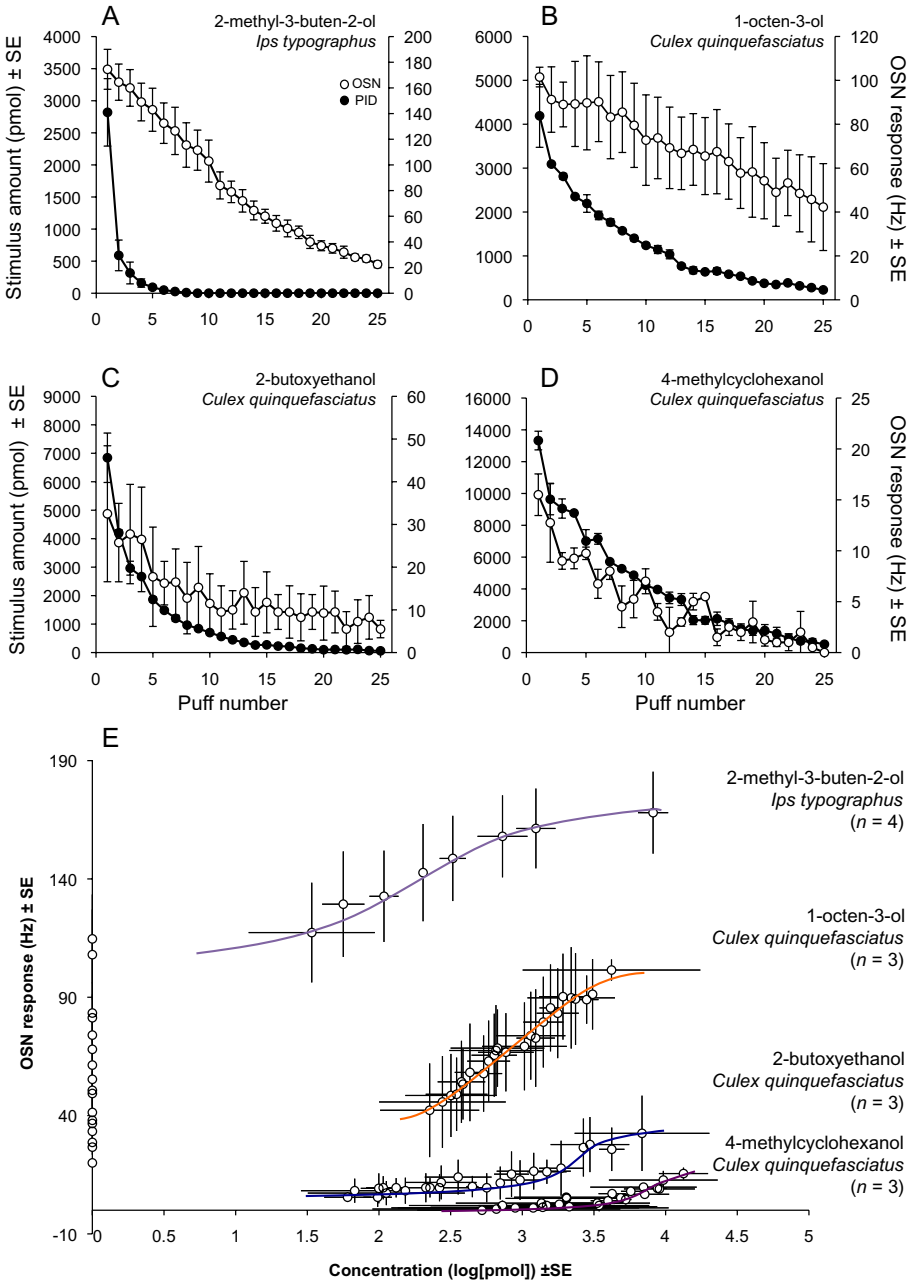

Figure 7 Responses of OSNs and the PID to successive stimulations of (**A**) MB in *Ips typographus* ($N = 4$) and (**B**) 1-octen-3-ol, (**C**) 2-butoxyethanol, and (**D**) 4-methylcyclohexanol in the *Culex quinquefasciatus* short sharp trichoid 3A neuron (SST3A; $N = 3$). Note the different scales for the stimulus amount and OSN response in **A**–**D**. (**E**) Dose–response curves based on airborne amounts of compounds for the 2 OSN types in the 2 species. Please note, this figure appears in color in the online version of *Chemical Senses*.

We disentangled some of the major factors contributing to the huge differences in airborne concentrations between olfactory ligands in standard stimulus pipettes: the chemical properties of the ligand, type of solvent, applied stimulus load, storage, and puff number. We further show that, for compounds diluted in paraffin, VP alone cannot be used as correction factor for volatilization rate and stimulus quantity. Instead, multiple regression analysis indicated that an empirical formula based on a set of physical characteristics (in our analysis: boiling point and lipophilicity) can be derived. Without such a formula, a PID with calibration factors is a very useful instrument for inline quantification of volatile compounds in physiological and possibly behavioral studies.

Successive stimulation

Studies generally do not detail the number of stimulations performed with a stimulus cartridge, or whether these are

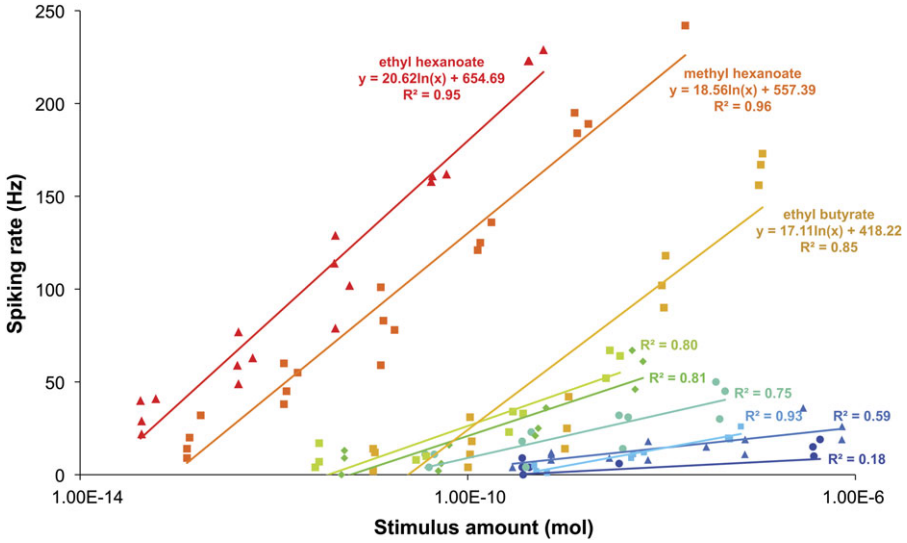

Figure 8 Dose–response curves of the AB3A neuron to airborne amounts of 9 ligands. Stimulations were performed using an MPS-2 autosampler. Derived equations (only for the 3 best ligands) and the R^2 values are displayed besides each regression. Please note, this figure appears in color in the online version of *Chemical Senses.*

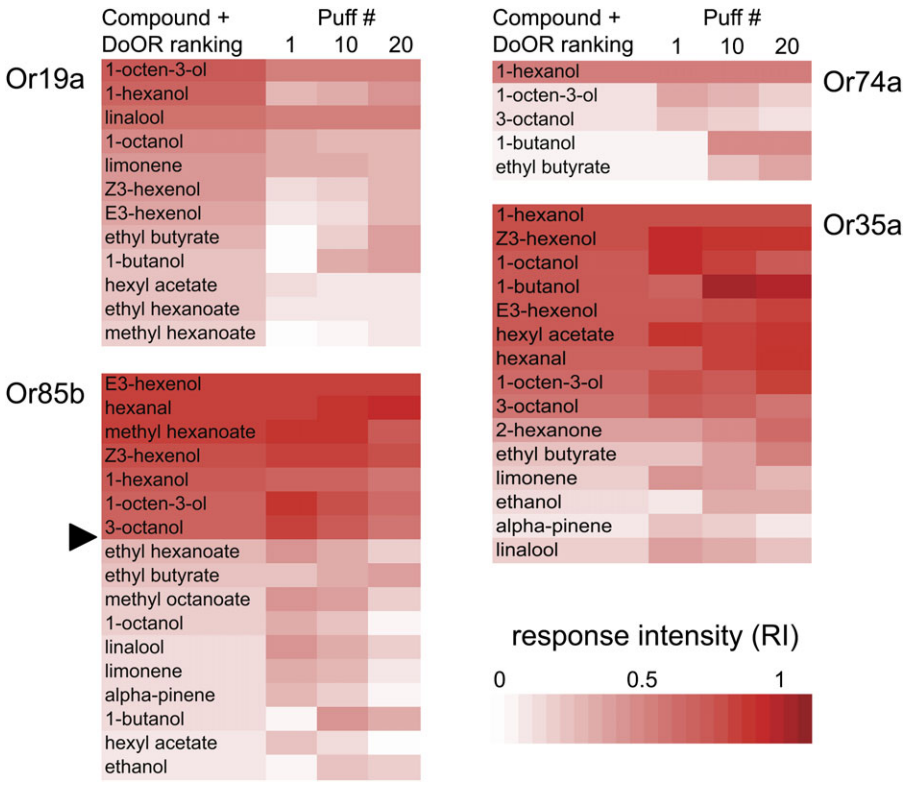

Figure 9 Comparison of data extracted from the DoOR database (Galizia et al. 2010) after compensation for headspace quantity for 4 *Drosophila melanogaster* Ors: Or19a, Or35a, Or74a, and Or85b. The studies underlying the DoOR data do not detail the number of stimulations a pipette has been used for. However, this factor affects the stimulus intensity strongly (this study). Therefore, we calibrated the DoOR data assuming they were based on the 1st, 10th, or 20th puff. The shading in the column containing the compound name (first column) reflects the RIs (0–1.0) derived from DoOR. The shade in the other columns indicates the calculated sensitivity of the neuron to that compound after correcting for airborne amount and puff number. Substantial shifts in sensitivities are found after correction (for details, see text and "Material and methods"). The arrowhead (Or85b) indicates that a few compounds with RIs (DoOR) in between those for 3-octanol and ethyl hexanoate were lacking in our test panel. Please note, this figure appears in color in the online version of *Chemical Senses.*

held constant across a panel of stimuli. With the solvent held constant (paraffin), a very significant factor that induces variation in headspace quantity between stimuli is repeated stimulation with the same stimulus pipette. Repeated stimulation can result in airborne quantities that differ by many log factors, depending on the compound. For instance, the ratio 1-butanol/1-hexanol dropped over a factor 100 within the first 10 puffs, and the ratio between 2-hexanone/1-hexanol dropped by a factor 20 in 10 puffs. Such variation may result in misclassification of sensory neurons, as demonstrated by our physiological recordings on *I. typographus* (Figure 6). Whereas some compounds showed a linear change in concentration with consecutive stimulations, others are no longer detected within a few stimulations and are best fitted on a log(stimulation #)–log(quantity) scale. Most compounds, however, fitted a log–linear model. In contrast, stimulation with pentane fitted the log–log model best, underlining the rapid decline in airborne concentration and thus the unreliability of stimulus concentration over successive stimulation.

In order to make later calibration of data possible, we recommend to puff a panel of odor cartridges an equal number of times, and to tag each physiological recording trace with the puff number. This permits later correction for vapor concentration.

Effect of solvent on evaporation and reliability of stimuli

Across laboratories different solvents are used for diluting odors. Alkane solvents and paraffin oil are frequently used, but other volatile solvents are also used, such as acetone, dichloromethane, and diethyl ether. The use of highly volatile solvents may be primarily for pragmatic reasons, as they are useful for making serial dilutions, for analysis via GC, and for the fact that they induce (after evaporation of the solvent) lower background responses in certain experimental setups (e.g., optical imaging). On the other hand, paraffin oil is used to get a more uniform stimulus quantity across stimulations. Thus, the type of solvent may affect vapor phase concentrations (Cometto-Muñiz et al. 2003).

In our study, we observed that all compounds dissolved in pentane were released at a much higher rate than in paraffin oil. This may come as no surprise, as paraffin acts as a slow-release matrix for organic stimuli. However, paraffin oil did not only reduce volatilization across the entire odor panel; the decrease in volatilization also differed between stimuli. Some compounds that were depleted extremely quickly when diluted in pentane (e.g., α-pinene, myrcene, and methyl octanoate) were depleted at a relatively low rate in paraffin, whereas for other compounds, the differences were not so large. The solvent-dependent relative depletion rates in the present study may be explained by differences in affinities for paraffin oil as compared with the filter paper (after the evaporation of pentane). Other highly volatile solvents, not tested here, likely would have given highly similar rates. Be that as it may, paraffin seems a vastly better choice of solvent than the very volatile ones, as it reduces variability between stimulations. Therefore, paraffin permits more accurate determination of headspace quantities and accordingly can be used in compensation for differences between stimuli.

Yet, paraffin may not be a one-fit-all solution. Certain odors cannot reliably be dissolved in paraffin (short-chain compounds with a strong polar moiety), whereas others might not be released reliably because of the high affinity with paraffin (long-chain compounds). We have no data for longer chain compounds due to insensitivity of the PID. However, a large variation in airborne amounts among heavier plant compounds and longer chain moth pheromones was recorded previously using quantification by means of a GC (Bengtsson et al. 1990; Todd and Baker 1993; Cossé et al. 1998).

Initial stimulus load

Airborne concentrations of stimuli are thought to be linearly related to the concentration applied on the stimulus cartridge, and dose–response studies are typically based on that assumption. Here, we demonstrate that, although 10-fold differences in the dose on the filter paper resulted in similar changes in the PID reading at the initial puff, the rate of depletion during subsequent puffing increased with decreasing doses (Figure 4; C6 alcohols). Possibly, this occurs through an increased resorption over desorption rate when volatiles accumulate in the headspace at high application doses. The fact that this did not hold true for MB may suggest a different relationship for highly volatile compounds. The data on stimulus load show, again, that measurements of headspace quantities need to be done in dose–response experiments instead of solely relying on the stimulus dose applied on the filter paper.

Headspace accumulation and stimulus storage

Laboratories differ in their practices of storing stimulus cartridges. Cartridges are prepared either freshly, prior to experiments, or used after storage in the freezer. In addition, most studies do not detail the interval between application of the stimulus and the start of the experiment.

Here, we show that at RT, headspace concentrations of the less volatile compounds (e.g., 3-octanol and methyl salicylate; Figure 5D,E) increased for up to approximately 2 h after loading, whereas those of highly volatile compounds (e.g., MB and *Z*3-hexenol; Figure 5A,B) were significantly depleted over time, even though the large aperture of the pipettes was capped. However, the reduction during the first 8 h, although significant, was minimal compared with the reduction seen due to reiterative stimulations. This implies that depletion and/or accumulation during storage are of little importance in studies where stimulus pipettes are used for successive stimulations, as long as the stimuli are not too volatile (e.g., MB). It remains unclear whether headspace

concentrations of compounds less volatile than tested here would accumulate more significantly over time. If that would be the case, then repetitive stimulation at different frequencies may cause variation in headspace quantity between puffs and thereby affect reproducibility of results.

At –20 °C, loaded pipettes kept most of the molecules, judged from the headspace concentrations, showing that freezing is a good method to store pipettes over longer time. However, cartridges containing very volatile compounds (e.g., MB) start to deplete significantly even at –20 °C, indicating that when using such volatiles, only short duration storage in the freezer warrants reproducible results.

VP and other explanatory variables

Surprisingly, in our study, VP, which is sometimes used to compensate for differential headspace concentrations (Hoskovec et al. 1993), was not a good predictor of compound depletion rates. First, log(VP) explained the depletion rates better than did untransformed VP. Second, and perhaps more importantly, we recorded substantial shifts between VP-predicted and experimental depletion rates in our odor panel (see shading in Table 1), which indicates a significant contribution of factors other than VP in volatilization. At closer scrutiny, the experimental rates deviated especially when VP-predicted rates were similar, that is, VP values may give an indication of volatilization rates when odors differ vastly in VP but fail to project headspace quantities when VP values are more similar. VPs are based on volatilization of a compound itself. Because we used paraffin oil together with filter paper as a slow-release cartridge, the interactions with solvent and cartridge are likely to cause deviation of volatilization from VP values. Indeed, Brockerhoff and Grant (1999), using hexane as solvent and a GC for quantification, found a better linear relation (R^2 = 0.89) than us between headspace concentration and VP of 5 monoterpenes, indicating that VP might be a better predictor if volatile solvents are used. Unfortunately, a comparison with our pentane test series is problematic because we had to discard the initial puff due to PID responses to the solvent. In contrast to volatile solvents, paraffin oil is good for slow and more constant release, but it makes correlation with VP values tenuous. However, the limited number of compound types tested by Brockerhoff and Grant (1999) makes it difficult to draw any general conclusions.

Because VP alone was an unsatisfactory predictor of depletion rate, we tested all independent factors in a multiple regression model. Boiling point and lipophilicity, but not VP, turned out as the factors that, when combined predicted the depletion rate best. We conjecture that further research, including a larger set of stimuli, could make it possible to derive an even better empirical equation that can be used by experimenters to correct for headspace quantities of stimuli.

Physiological verification

We verified how differential volatilization could affect physiological characterization and how useful a PID could be in providing correction factors for such physiological studies. The results show that quantification of stimuli is important for characterization of the olfactory sensory array of an insect, for analyses of ligand-OSN interactions, and for dose–response relationships. Furthermore, using a PID, one can derive absolute sensitivities and compare these across different OSNs and odors.

Characterization of OSN arrays

Efforts mapping out OSN types in the olfactory tissue sometimes rely on subtle differences in tuning curves between neuronal types. Differences in headspace concentrations could lead to misidentification in assignment of key ligands or to misclassification of OSN types.

First, our recordings on a "generalist" neuron, the AB3A OSN in *D. melanogaster*, demonstrate that the key ligands assigned to a neuron may shift after compensation for headspace quantities. Various studies report that the AB3A neuron and its cognate olfactory receptor, Or22a, are most sensitive to methyl hexanoate and ethyl hexanoate, but studies provide conflicting results regarding which of the 2 is the best ligand (Stensmyr et al. 2003; Hallem and Carlson 2006; Pelz et al. 2006). Using absolute quantification, we demonstrate that the AB3A neuron is approximately 10-fold more sensitive to ethyl hexanoate than to methyl hexanoate.

Second, misclassification of OSNs due to differences in headspace quantities between successive stimulations can lead to an inflated number of characterized OSN types, especially if generalist OSNs are involved. This is exemplified by our mapping effort on the bark beetle (Andersson et al. 2009), in which 1 OSN type could easily be mistaken for 2 due to different RIs of the OSN to fresh and previously used stimulus cartridges (Figure 6). Along with the identification of Ors in a diverse set of insects, mapping of OSNs is conducted on numerous species. OSN types described should be carefully scrutinized in the light of the above and, where possible, corrected for headspace quantities.

OSN tuning curves and stimulus purity

Using headspace quantification, we also demonstrate that the concept of generalist and specialist neurons is partially a matter of concentration. In previous work on the *C. quinquefasciatus* SST3A neuron, 1-octen-3-ol and 2-butoxyethanol elicited similarly strong responses, whereas 4-methylcyclohexanol elicited responses that were approximately 50% weaker (10% stimulus load) (Hill et al. 2009). However, after calibration with airborne concentrations, the neuron turned out to be approximately 10× more sensitive to 1-octen-3-ol than to either of the other 2 alcohols. Similarly, the AB3A neuron of the fruit fly is sensitive to femtomolar quantities of ethyl hexanoate (Dekker et al. 2006), with other "key" ligands many log values

less potent (except for methyl hexanoate). A generalist OSN may thus ecologically be a specialist, depending on the naturally encountered concentration of ligands. In contrast, OSNs responding to carboxylic acids in *Anopheles gambiae* were equally sensitive to acids with different chain lengths only after correction for differences in volatility (Meijerink and van Loon 1999). Dose–response studies often indicate that generalist neurons are considerably more narrow in their sensitivity at lower dose (e.g., Ghaninia et al. 2007; Andersson et al. 2009; Bengtsson et al. 2009; Hill et al. 2009; Carey et al. 2010). However, very few studies have tried to elucidate which doses are within the ecologically relevant range.

On a similar note, our data underline the importance of purity in characterization studies. The *D. melanogaster* AB3A neuron did not show a pronounced response to some ligands that reportedly induce responses of similar strength as the hexanoates, for instance, methyl octanoate. This indicates that, in our study, much lower concentrations of these compounds were used than in other studies (see, e.g., DoOR library). However, with such vast difference in sensitivity, spanning many log concentrations, the purity of stimuli may be an issue. Because the AB3A neuron responds to ethyl butyrate at 4 orders of magnitude higher concentration than to ethyl hexanoate, contamination of ethyl butyrate with trace amounts (0.01%) of ethyl hexanoate could underlie the response of the AB3A neuron to ethyl butyrate. The situation is worse for less strong ligands, such as methyl octanoate. Such trace amounts may not be reliably detected by GC analysis alone and would necessitate GC-SSR analysis to verify if the sensory response is induced by the presumed ligand or by a contaminant. It would thus be advisable to carry out such analyses routinely, as part of ongoing OSN mapping efforts.

Detection thresholds of OSNs

Quantification of stimuli also permits an analysis of the absolute sensitivity of OSNs and an analysis of the factors that contribute to this. For example, AB3A neurons in *D. melanogaster*, MB-sensitive neurons in *I. typographus* and SST3A neurons in *C. quinquefasciatus* are several orders of magnitude more sensitive to their respective key ligands than is the PID. Among those, the *D. melanogaster* AB3A neuron is by far the most sensitive, followed by the *I. typographus* MB neuron, and finally the mosquito SST3A neuron. Finally, it appeared that the *I. typographus* 3-octanol neuron is several orders of magnitude more sensitive to 1-octen-3-ol than is the mosquito SST3A neuron, for which 1-octen-3-ol is assigned the key ligand. The question is what factors contribute to these vast differences in sensitivity between species. Quantification of headspace amounts is the first necessary step in determining which factors that may underlie such differences.

Besides highlighting shifts in OSN affinities, our quantification of stimuli also sheds some light on an issue that arose from our previous bark beetle study. The fact that the bark beetle OSN still responded to MB after 50 puffs (25 into PID, 25 onto antenna) indicates that the low number of reported MB cells (Andersson et al. 2009) is not the result of the compound being depleted from the pipette, but rather the result of the distribution of these cell types in a restricted area on the antenna.

Retroactive dose–compensation of DoOR data

To verify the potential significance of volatilization and depletion rates, we extended our analysis to already published data on *D. melanogaster* Ors (DoOR) (Galizia et al. 2010). These show that under the assumption of similar slopes for the regression curves across stimuli and a similar number of stimulations per cartridge, one can expect shifts in ligand hierarchy (the sorting of stimuli from best to worst ligand affinity). The precise hierarchy furthermore depends on the number of stimulations per cartridge. This implies that, as long as there is a good record of the stimulation number, one could calibrate data sets for headspace quantities, with the possibility of doing so also retroactively.

Further analysis demonstrates that, although databases such as DoOR are extremely valuable, they should be complemented with dose–response data for more accurately assessing ligand affinities. To illustrate this, our study on the AB3A neuron clearly demonstrates that ethyl hexanoate is by far the best ligand tested, followed by methyl hexanoate and ethyl butyrate. Methyl octanoate induced only weak responses. In contrast, the DoOR database assigns higher affinities of Or22a (expressed in the AB3A OSN) to methyl octanoate than either methyl hexanoate or ethyl butyrate. It may be that the slope of methyl octanoate is very different from the other 2 ligands (see Figure 8; differences in dose–response slopes exist between key and nonkey ligands) or there may be a contaminant in the methyl octanoate responses that were used in the DoOR database. Be that as it may, it is important that the DoOR and similar databases are further compensated for with headspace quantities and extended with dose–response data to maximize their usability.

Conclusions and take home message

In conclusion, we demonstrate that airborne amounts emitted from stimulus pipettes vary greatly, which can affect conclusions drawn in chemosensory research in terms of ligand affinity and tuning breadth of Ors and OSNs. This underlines the need for an instrument that can provide fast and direct measurements of the vapor phase or the need for alternative odor delivery systems that provide better control of the stimuli, for example, the autosampler used here or the system described in Olsson et al. (2010).

In addition, our results suggest that it is, with not too much effort, possible to follow the next rules:

(I) Compensate for differences in vapor quantity.
(II) Practice consistent handling of stimulus pipettes.
(III) Use volatile stimuli for only a restricted number of stimulations.
(IV) Store pipettes only for restricted periods and at low temperature.
(V) Use the same solvent, preferably paraffin oil.
(VI) Wherever possible, complement data with GC-coupled SSR to avoid contaminants affecting conclusions.
(VII) Finally, researchers should carefully tag physiological recordings with the number of times a cartridge has been used, as this permits retroactive calibration of data sets.

The PID proved to be useful for measurements of airborne concentrations for more volatile compounds (C2–C10), and it should be affordable for most labs.

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